Introduction

The development of new vaccine against tuberculosis (TB) is essential to protect many people from the infection of M.tuberculosis in the world. New vaccine is also necessary to cure the patients with MDR-TB. Therefore, the development of new vaccine was studied using three distinct methods; 1. DNA 2. rBCG and 3. subunit vaccination. In our previous report, IFN-γ gene and IL-6 related gene (IL-6 gene + IL-6R gene + gp130 gene) using adenovirus vector exerted significant efficacy against TB(1). Cytotoxic T lymphocyte (CTL) is suggested to play an important role in the anti-tuberculosis immunity in mice vaccinated related genes as well as tumor immunity (1-4). In the present paper, HVJ-liposome / HSP65 DNA+ IL-12 DNA or r72f BCG showed the prophylactic effect in the cynomolgus monkey as well as mice challenged with M.TB.

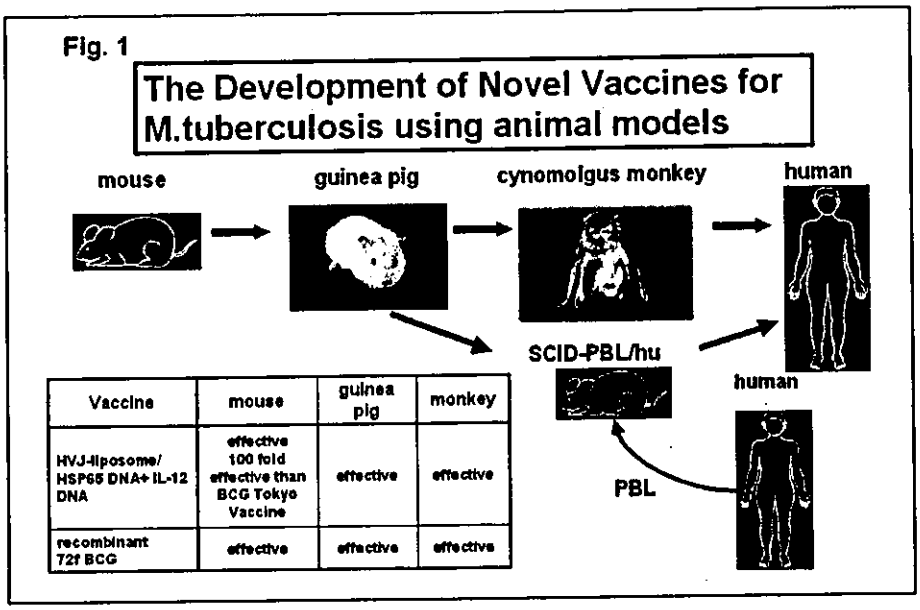
Materials and Methods

IL-12 gene or heat shock protein (HSP65) gene derived from M.TB was constructed as DNA vaccine into plasmid using CMV promoter. DNA vaccination into female BALB/c (8 weeks, CEA Japan Inc., Japan) was performed using HVJ-liposome CTL activity was assessed by the detection of IFN-γ activity in the culture supernatant of 20 hrs. culture consisting effector spleen cells from vaccine(s)-treated mice in the presence of J774.1 macrophage cells pulsed with M.TB. Cynomologus monkeys were immunized three times with (1)r72f BCG vaccine (2)HVJ-liposome / HSP65 DNA + IL-12 DNA vaccine (3)72f fusion protein + BCG Tokyo (4)BCG Tokyo (5)Saline. One month after last immunization, M.TB (Erdman strain $5X10^2$) were challenged by intratracheally instillation. BSR, body weight, chest X-P, immune responses, DTH reaction against PPD, and survival were studied.

Results

An infection initiated by intravenous injection of virulent M.TB H37RV ($5X10^5$) was allowed to develop for 10 weeks, the duration time of which the number of bacteria in the internal organs such as lung, liver, spleen increased. 10 weeks after challenge, mice coimmunized with HSP65 DNA vaccines and IL-12 expression vector had significantly reduced numbers of CFU in the all three organs (lungs, liver, spleen) as compared with mice immunized with Mycobacterium bovis BCG vaccine. (Fig. 1) Spleen cells from the mice vaccinated with HSP65 plus IL-12 DNA produced more IFN-γ than those from the mice vaccinated with BCG Tokyo in the assay for CTL activity. Thus CTL activity correlated with the efficacy of vaccination.

Fig. 1



Vaccine	mouse	guinea pig	monkey
HVJ-liposome/ HSP65 DNA+ IL-12 DNA	effective 100 fold effective than BCG Tokyo Vaccine	effective	effective
recombinant 72f BCG	effective	effective	effective

Fusion protein Mtb72f (Mtb39 + Mtb32) vaccine was developed by Dr. Steven Reed et al, Corixa. To develop more strong vaccination, rBCG secreting 72f was constructed using 72f fusion gene. R72f BCG exerted stronger effect on the increase in the number of IFN-γ producing T cells than BCG Tokyo in mice by ELISPOT. In guinea pig model, r72f BCG as well as HSP65 + IL-12 DNA vaccine improved the histopathological observations of the lungs.

Survival of monkeys vaccinated with HSP65 + IL-12 DNA vaccine was better than that of control (saline) monkeys. (Table 1) All 4 monkeys of control saline groups died of TB infection. In contrast, 2 monkeys out of 4 in the HSP65 + IL-12 DNA group were alive more than 14 months. Survival of monkeys vaccinated with r72f BCG was better than that of BCG vaccine group. All 4 monkeys in the 72f fusion protein + BCG Tokyo vaccine group were alive. HSP65 + IL-12 DNA vaccine and r72f BCG vaccine improved ESR and chest X-P finding and induced the increase in weight of TB- infected cynomolgus monkeys. The augmentation of IFN-γ production was significantly augmented in the monkeys immunized with these three kinds of vaccines. IL-2 production and proliferation of PBL were strongly enhanced in the monkeys vaccinated with HSP65 + IL-12 DNA vaccine.

Conclusions

(1)Novel HVJ-liposome / HSP65 DNA + IL-12 DNA vaccine showed

Table 1

The Development of Novel vaccines using cynomolgus monkey Anti-tuberculosis effect of ① HSP65DNA+IL-12 DNA vaccine, ② recombinant 72f BCG vaccine

Three distinct kind of TB vaccines	Prophylactic effect against M.tb	survival	Improvement of BSR	Increase in Body weight	Improvement of Chest X-ray finding	Immune Responses		
						Proliferative responses of lymphocyte	IFN-γ production	IL-2 production
① HVJ-liposome /HSP65 DNA + IL-12 DNA vaccine	++	++	++	+	+	+++	+	+
② recombinant 72f BCG vaccine	++	++	+	+	+	+	+	+

prophylactic effect on TB infection using murine, guinea pig and cynomolgus monkey models. (2)HSP65 + IL-12 DNA vaccination was more efficient (100 fold) than parental BCG Tokyo vaccination in mice. CTL activity against M.TB was augmented. (3)By using new vaccines (HSP65 DNA + IL-12 DNA and r72f BCG) and the cynomolgus monkey models which are very similar to human tuberculosis, the significant prophylactic effect of vaccines are observed. (4)HSP65 + IL-12 DNA vaccine, r72f BCG vaccine and (72f fusion protein + BCG Tokyo) vaccine (a)Prolonged the survival time (b)Improved the BSR, chest X-P findings, and immune responses.

The SCID-PBL/hu mouse model, which in capable of analyzing in vivo human immune responses, was also used for theses studies because it is a highly relevant translational for human cases. (4) (Fig. 1) We plan to do clinical trial using, HVJ-Envelope / HSP65 + IL-12DNA vaccine.

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Specific Detection of Tuberculosis Infection

An Interferon-γ–based Assay Using New Antigens

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The tuberculin skin test for immunologic diagnosis of *Mycobacterium tuberculosis* infection has many limitations, including being confounded by bacillus Calmette-Guérin (BCG) vaccination or exposure to nontuberculous mycobacteria. *M. tuberculosis*–specific antigens that are absent from BCG and most nontuberculous mycobacteria have been identified. We examined the use of two of these antigens, CFP-10 and ESAT-6, in a whole blood IFN-γ assay as a diagnostic test for tuberculosis in BCG-vaccinated individuals. Because of the lack of an accurate standard with which to compare new tests for *M. tuberculosis* infection, specificity of the whole blood IFN-γ assay was estimated on the basis of data from people with no identified risk for *M. tuberculosis* exposure (216 BCG-vaccinated Japanese adults) and sensitivity was estimated on the basis of data from 118 patients with culture-confirmed *M. tuberculosis* infection who had received less than 1 week of treatment. Using a combination of CFP-10 and ESAT-6 responses, the specificity of the test for the low-risk group was 98.1% and the sensitivity for patients with *M. tuberculosis* infection was 89.0%. The results demonstrate that the whole blood IFN-γ assay using CFP-10 and ESAT-6 was highly specific and sensitive for *M. tuberculosis* infection and was unaffected by BCG vaccination status.

Keywords: bacillus Calmette-Guérin; diagnostics; infection; IFN-γ; tuberculosis

Tuberculosis continues to be a heavy burden on human health, with the World Health Organization estimating that one-third of the world's population is infected with *Mycobacterium tuberculosis* (1). Detection and treatment of latent tuberculosis infection are important measures in the fight against this epidemic, especially in industrialized countries. The tuberculin skin test (TST) has been the only practical means of detecting latent *M. tuberculosis* infection in the past century. Unfortunately, the TST suffers from a number of well-documented performance and logistic problems, the most serious being false-positive responses due to reactivity caused either by infection with nontuberculous mycobacteria (NTM), or by bacillus Calmette-Guérin (BCG) vaccination (2, 3).

An *in vitro* whole blood test that detects *M. tuberculosis* infection by measuring IFN-γ responses to tuberculin purified protein derivative (PPD) was approved in the United States. Although this assay may be less affected by BCG vaccination than the TST (3), it is falsely positive in some BCG-vaccinated individuals (4) as many PPD antigens are similar or identical to antigens in BCG and NTM. Parts of the *M. tuberculosis* genome that are absent from the genomes of all BCG substrains and most NTM have been identified (5). These *M. tuberculosis*–specific regions encode a number of proteins including CFP-10 and ESAT-6. Cell-mediated responses to these antigens have been shown to correlate with both proven *M. tuberculosis* infection and a high risk of infection (4, 5–10). The application of CFP-10 and ESAT-6 to the whole blood IFN-γ assay should allow specific and sensitive diagnosis of *M. tuberculosis* infection in a relatively simple test format.

Thus, the aim of this study was to estimate the specificity and sensitivity of a whole blood IFN-γ assay employing CFP-10 and ESAT-6, for the detection of *M. tuberculosis* infection in a predominantly BCG-vaccinated population. Estimates of sensitivity and specificity of tests for *M. tuberculosis* infection are hampered by the lack of a "gold standard"; one cannot prove the presence or absence of latent tuberculosis (TB) infection. In this study, sensitivity was determined in untreated patients with culture-proven tuberculosis, which although definitive for active tuberculosis requires extrapolation to equate to latent tuberculosis infection. Specificity was estimated in a group of BCG-vaccinated individuals with no known risks for *M. tuberculosis* exposure.

METHODS

Participants

Patients and student nurses consenting to the study were enrolled in Tokyo (National Tokyo Hospital, Fukujuji Hospital, and Japan Anti-Tuberculosis Association), Osaka (National Kinki Chuo Hospital and Osaka Prefectural Habikino Hospital), Chiba (National Chiba Higashi Hospital; and Nursing College, Chiba University), Miyazaki (Miyazaki Prefectural Nursing University), and Hiroshima (National Hiroshima Hospital), Japan after the protocol was approved by each institution's ethics review committee. Subjects were enrolled into one of two groups: Group 1 consisted of student nurses (older than 17 years of age) who were enrolled at the beginning of their training and had no identified risk for *M. tuberculosis* exposure; and Group 2 consisted of patients clinically suspected to have active tuberculosis and who had received less than 1 week of antituberculosis treatment.

After giving written consent, subjects were asked to complete a questionnaire about possible risk factors for exposure to *M. tuberculosis*. For low-risk subjects enrolled into Group 1, data were collected on their country of birth, history of prior tuberculosis or exposure to a person with tuberculosis, and other tuberculosis risk factors such as having an immunosuppressive condition (i.e., human immunodeficiency virus [HIV], leukemia, lymphoma, diabetes mellitus, or renal failure) or having taken immune suppressive drugs in the 3 months before enrollment. Information regarding any previous Mantoux TST results and BCG vaccination status was also collected. For patients recruited into Group 2, information on their clinical symptoms of active tuberculosis and chest

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X-ray findings were collected at the time of enrollment. Sputum or other appropriate nonrespiratory samples were collected from Group 2 patients and cultured for mycobacteria.

Sample Collection and TST

A heparinized blood sample was collected for the whole blood IFN-γ assay from each subject by venipuncture. Blood was collected before administration of Mantoux TSTs when the latter test was performed. For the TST, 0.1 ml of tuberculin PPD (Nippon BCG Manufacturing, Tokyo, Japan; equivalent to about 3 TU of PPD-S) was injected intradermally into the volar aspect of the forearm and transverse induration diameter was measured 48 hours later.

M. tuberculosis–specific Antigens

Pools of overlapping peptides representing CFP-10 and ESAT-6 were used as TB-specific antigens in the whole blood IFN-γ assay. The sequence of six peptides representing CFP-10 and of seven peptides representing ESAT-6 are shown in Table 1. Peptides, manufactured by either Mimotopes (Clayton, Australia) or Schafer-N (Copenhagen, Denmark), were at least 79% pure as determined by HPLC analysis. Peptides were solubilized in phosphate-buffered saline and aliquots (10 μg/ml for each peptide) were stored at −70°C, before use in the whole blood IFN-γ assay.

Whole Blood IFN-γ Assay

The whole blood IFN-γ assay (QuantiFERON [QFT]; Cellestis, Carnegie, Australia) involves two stages: (*1*) overnight incubation of whole blood with antigens and (*2*) measurement of IFN-γ production in harvested plasma samples by ELISA. Within 12 hours of collection, 1-ml aliquots of blood samples were dispensed into 24-well tissue culture plates and antigens were added to appropriate wells. Three drops of saline (nil control) or phytohemagglutinin (5 μg/ml; mitogen-positive control), and 100 μl of ESAT-6 or CFP-10 peptide cocktail, were added to separate wells to give a final peptide concentration of 1 μg/ml. Blood samples were incubated with antigens for 16 to 24 hours at 37°C before harvesting about 300 μl of plasma from above the settled blood cells.

The concentration of IFN-γ in the four plasma samples from each subject was determined by QuantiFERON-CMI ELISA as per the manufacturer's instructions. This ELISA is reported by the manufacturer to have a limit of detection of 0.05 IU/ml for IFN-γ. Samples from up to 16 subjects were tested in each ELISA run, which also included a set of standards that were measured in duplicate. For an ELISA run to be valid, strict performance criteria (coefficient of variation less than 15% and correlation coefficient for the standard curve greater than 0.98) had to be met. ELISA data for the *M. tuberculosis*–specific antigens CFP-10 and ESAT-6 and the nil and mitogen controls were converted to international units per milliliter on the basis of the IFN-γ standard curve generated for each ELISA plate. For an individual's test to be deemed valid, their response to at least one antigen (ESAT-6, CFP-10, or mitogen) had to be at least 0.25 IU of IFN-γ per milliliter above that of their nil control (five times the limit of detection for the ELISA). Results for ESAT-6 and CFP-10 are expressed as the concentration of IFN-γ detected minus the concentration of IFN-γ in the respective nil control plasma.

TABLE 1. AMINO ACID SEQUENCES OF OVERLAPPING PEPTIDES FOR ESAT-6 AND CFP-10

Antigen	Amino Acid Sequence
CFP-10	
Peptide 1	MAEMKTDAATLAQEAGNFERISGDL
Peptide 2	GNFERISGDLKTQIDQVESTAGSLQ
Peptide 3	DQVESTAGSLQGQWRGAAGTAAQAAV
Peptide 4	AAGTAAQAAVVRFQEAANKQKQELD
Peptide 5	AANKQKQELDEISTNIRQAGVQYSR
Peptide 6	IRQAGVQYSRADEEQQQALSSQMGF
ESAT-6	
Peptide 1	MTEQQWNFAGIEAAASAIQG
Peptide 2	GIEAAASAIQGNVTSI
Peptide 3	SAIQGNVTSIHSLLDEGKQSLTKLA
Peptide 4	EGKQSLTKLAAAWGGSGSEAYQGVQ
Peptide 5	SGSEAYQGVQQKWDATATELNNALQ
Peptide 6	TATELNNALQNLARTISEAGQAMAS
Peptide 7	NLARTISEAGQAMASTEGNVTGMFA

Statistical Analysis

Information from the questionnaires, TST results, and whole blood IFN-γ assay results was entered into Excel 2000 (Microsoft, Redmond, WA) and transferred to Stata version 7.0 (Stata, College Station, TX) for statistical analysis. Analysis consisted of *t* tests for differences in means based on logarithmic transformation of the IFN-γ measurements, χ^2 test for testing difference in proportions, exact binomial methods to compute confidence intervals for proportions, and maximum-likelihood logistic regression to estimate the strength of the relation between age and response to the whole blood IFN-γ assay and the TST.

RESULTS

Subjects were enrolled into the study over a 4-month period from July to October 2002. There were 216 people with no identified risk for *M. tuberculosis* exposure enrolled into Group 1 and 152 tuberculosis suspects enrolled into Group 2. The mean age for Group 1 subjects was 20 years (range, 18–33 years) and for Group 2, 54 years (range, 13–86 years; age was not recorded for eight people). Group 1 subjects were predominantly female (92.7%), whereas Group 2 subjects were predominantly male (66.4%). No subjects in Group 1 reported any history of contact with patients with tuberculosis or of working in any health care setting.

The majority of Group 1 subjects had last been screened with the TST when entering junior high school, 6 years before the current study. None of these subjects reported having an immunosuppressive condition such as HIV, leukemia, lymphoma, diabetes mellitus, or renal failure; and none reported having taken immune-suppressive drugs in the 3 months before enrollment. TST results were available for 113 of the 216 Group 1 subjects; of them, 97 (85.8%) had an induration 5 mm or more, 73 (64.6%) had an induration 10 mm or more, and 36 (31.9%) had an induration 15 mm or more. Thus, taking 10-mm induration as the cutoff, the specificity of tuberculin skin testing was 35.4%. The mean age and its standard error of those without TST were 19.5 years and 0.266, which compared with those with TST (19.2 and 0.238, respectively). All Group 1 subjects reported having received BCG vaccination at least once by the time of graduation from junior high school.

Of the 152 TB suspects in Group 2, 119 were proven to have *M. tuberculosis* infection (and active tuberculosis) by culture of the organism from sputum or other bodily samples. Sputum acid-fast smear results were available for only 78 of the 119 persons with culture-proven tuberculosis, as one hospital did not report smear results. Sixty-eight of 78 patients had positive smears. One person, whose culture was positive for *M. tuberculosis*, had an indeterminate QFT result due to insufficient IFN-γ production in response to the mitogen or TB-specific antigens. Results from this person were omitted from further analysis. *M. tuberculosis* was recovered from pleural fluid of four Group 2 subjects and from sputum of 114 subjects. All TB suspects had received less than 7 days of antituberculous chemotherapy at the time of testing; 95 (80.5%) had received none. TST results were available for 76 of the 118 evaluable Group 2 subjects; 50 of these (65.8%) displayed an induration of 5 mm or greater. The patients who had TST results had a mean age (± standard error) of 54.7 ± 2.3 years, compared with 51.7 ± 3.6 years for those in whom skin tests were not performed ($p = 0.74$). Both groups had a similar sex distribution (65 and 66% males, respectively; $p = 0.96$) and a similar percentage of patients with positive sputum acid-fast smears (92 and 82%, respectively; $p = 0.17$).

No patients self-reported to be seropositive for HIV, undergoing hemodialysis, currently being treated with corticosteroids, or known to have a malignant disease. There were four patients with diabetes mellitus. There were 33 people in Group 2 whose cultures were negative for *M. tuberculosis* despite symptoms and suspicion of active tuberculosis; *Mycobacterium avium* complex (MAC) organisms were recovered from 5 of these people; *Mycobacterium kansasii* was recovered from 3; and 25 had negative culture results for mycobacteria.

Response to Specific Antigens

All IFN-γ ELISA runs met the specified performance criteria and were deemed valid. The range of responses in the whole blood IFN-γ assay for subjects in each study group are shown in Figure 1. Patients with culture-proven tuberculosis had a significantly higher mean IFN-γ response than did low-risk Group 1 subjects for both CFP-10 (geometric means being 0.657 and 0.010 IU/ml, respectively; $p < 0.001$) and ESAT-6 (1.330 and 0.003 IU/ml, respectively; $p < 0.001$).

Table 2 shows test specificities and sensitivities for CFP-10 and ESAT-6 at various cutoff concentrations. To estimate specificity, all 216 subjects in Group 1 were assumed not to be infected with *M. tuberculosis*. To estimate sensitivity, only QFT results from the 118 Group 2 subjects for whom *M. tuberculosis* infection was confirmed by culture were used. To ascertain appropriate cutoffs for the ESAT-6 and CFP-10 antigens, receiver operating characteristic analysis was performed, based on data from Group 1 individuals for specificity and Group 2 patients with culture-confirmed *M. tuberculosis* infection for sensitivity. Receiver operating characteristic analysis was performed with data from these subjects and confirmed that 0.35 IU/ml was an appropriate cutoff for both CFP-10 and ESAT-6. This cutoff was chosen to maximize specificity without significant loss of test sensitivity. Using this cutoff, the specificities (with 95% confidence intervals) for CFP-10 and ESAT-6 were 98.6% (96.0 to 99.7%; n = 213, data for CFP-10 were unavailable for three people because of insufficient blood being collected) and 99.5% (97.5 to 100.0%; n = 216), respectively, and the sensitivities were 65.3% (55.9 to 73.8%) and 81.4% (73.1 to 87.9%), respectively. If the data from CFP-10 and ESAT-6 were combined such that a person positive to at least one of the two antigens is judged as test positive, a sensitivity of 89.0% (81.9 to 94.0%) and a specificity of 98.1% (95.3 to 99.5%; n = 213) were obtained.

Test results were positive in 60 (88%) of 68 patients with positive sputum acid-fast smears and 6 (60%) of 10 patients with negative smears ($p = 0.07$).

Data for the 33 people in Group 2 whose cultures were negative for *M. tuberculosis* despite symptoms and suspicion of active tuberculosis are shown in Figure 1C. For the 25 tuberculosis suspects from whom mycobacteria were not recovered, 56% (14) were positive to either CFP-10 or ESAT-6 in the whole blood IFN-γ assay, a significantly smaller proportion as compared with those with culture-confirmed *M. tuberculosis* infection (89%; χ^2 test, $p = 0.0001$). The whole blood IFN-γ assay with either antigen was positive for all three patients from whom *M. kansasii* was recovered. For one of the five patients from whom MAC was recovered, the CFP-10 response was positive (IFN-γ, 7.5 IU/ml).

To examine the effect of age on sensitivity of the whole blood IFN-γ assay and the TST, data from the 110 patients with confirmed tuberculosis, and whose ages were recorded, were stratified as shown in Table 3. Logistic regression analyses were used to estimate the associations between age and QFT response, and between age and TST response. On average, persons were 0.83 times as likely to have a positive QFT and 0.71 times as likely to have a positive TST, compared with persons 10 years younger. The 95% confidence interval for the former odds ratio was 0.56 to 1.23, with decline being not statistically significant ($p = 0.35$), and that for the latter was 0.53 to 0.94, with a statistically significant decline ($p = 0.015$).

DISCUSSION

The current study demonstrates a high degree of accuracy in detecting *M. tuberculosis* infection, using the whole blood IFN-γ assay with the *M. tuberculosis*–specific proteins CFP-10 and ESAT-6. The assay was shown to be highly specific (greater than 98%) in BCG-vaccinated low-risk subjects (Group 1) assumed to be truly free of *M. tuberculosis* infection. Specificity of the whole blood IFN-γ assay was much better than the specificity observed for the TST in the present study (35.4%, using a 10-mm induration cutoff), or previously reported for Japan (10%) (11). Although we assumed that none of the Group 1 subjects were infected with *M. tuberculosis*, it is probable that some of the 216 subjects had been infected, as the prevalence of *M. tuberculosis* infection in 20-year-old people in Japan is estimated at 1% (12).

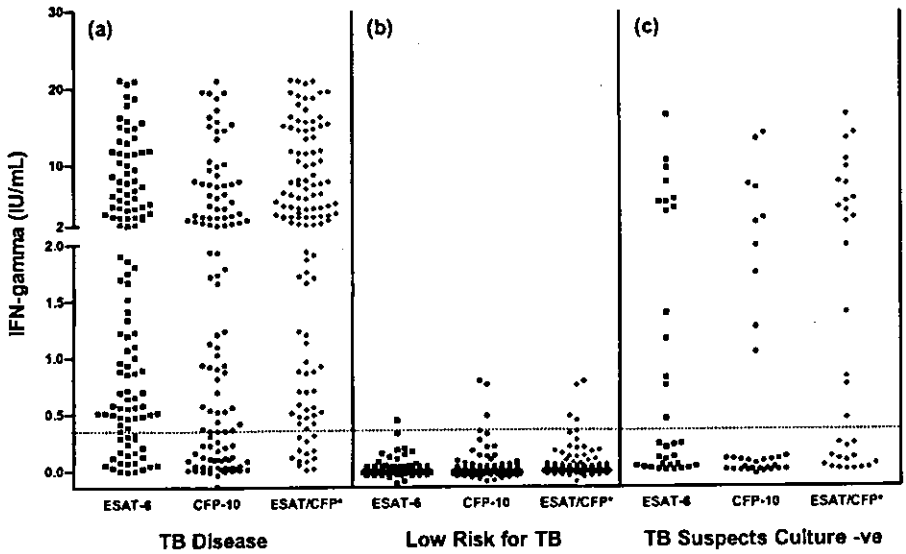

Figure 1. Dot plot of individual responses to CFP-10 and ESAT-6 for 118 culture-positive patients with tuberculosis (TB) (*a*), 213 subjects with a low risk for TB exposure (*b*), and 33 TB suspects whose TB status could not be determined, as *Mycobacterium tuberculosis* could not be cultured (*c*). *For "ESAT/CFP" the data for the antigen (ESAT-6 or CFP-10) giving the highest response is shown. The *dashed line* represents the cutoff of 0.35 IU/ml for IFN-γ.

TABLE 2. TEST SENSITIVITY AND SPECIFICITY FOR CFP-10 AND ESAT-6 AT VARIOUS CUTOFFS IN WHOLE-BLOOD IFN-γ ASSAY

Cutoff, IFN-γ	CFP-10		ESAT-6		CFP-10 and/or ESAT-6	
(*IU/ml*)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)	Sensitivity (%)
0.05	92.5	81.4	94.8	94.9	89.4	97.5
0.10	94.4	77.1	96.2	90.7	92.0	95.8
0.15	95.8	72.9	97.6	88.1	93.9	93.2
0.20	96.7	71.2	99.1	86.4	96.2	91.5
0.25	97.2	67.8	99.1	84.7	96.7	91.5
0.30	97.7	66.9	99.1	83.1	97.2	89.8
0.35	**98.6**	**65.3**	**99.5**	**81.4**	**98.1**	**89.0**
0.40	98.6	61.9	99.5	79.7	98.1	88.1
0.45	98.6	60.2	100.0	78.8	98.6	86.4
0.50	99.1	60.2	100.0	75.4	99.1	83.9

Sensitivity was determined on the basis of data from 118 patients with culture-positive tuberculosis, and specificity was determined on the basis of data from 213 low-risk subjects. The chosen cutoff (0.35) is in boldface.

Thus, the true specificity of the test may be higher than that estimated in the present study.

To estimate sensitivity of the whole blood IFN-γ test, the presence of culture-confirmed *M. tuberculosis* infection was used as the standard. This approach has been widely used in sensitivity studies with the TST, often using patients who were receiving, or who had completed, treatment at the time of testing (3, 13–16). However, as it is well documented that both IFN-γ responses can vary in relation to antituberculosis treatment (3, 17–19), we limited this study to patients who had received minimal or no treatment at the time of testing. At the time of enrollment into the study, all 152 Group 2 subjects had radiologic and/or clinical signs suggesting tuberculosis and sensitivity was estimated from the 118 who had *M. tuberculosis* recovered subsequently by culture. Both ESAT-6 and CFP-10 demonstrated high positive rates in these patients (65.3 and 81.4%, respectively) as compared with that in tuberculin skin testing (65.8%). Combining results from the *M. tuberculosis*–specific antigens improved test sensitivity to 89.0% and had little effect on specificity (98.1%).

The poor skin test specificity of TST (35.4%) seen in this study is likely to be predominantly a result of the extensive use of BCG vaccination in Japan. However, poor skin test specificity may also be due to exposure or infection with NTM. Exposure to NTM, and not latent *M. tuberculosis* infection, appears to be responsible for the majority of 5- to 14-mm Mantoux test reactions among U.S.-born health care workers and medical students (20). The present study was not designed to assess the specificity of the whole blood IFN-γ assay after exposure to NTM. However, given the reported mycobacterial species specificity of ESAT-6 and CFP-10 (5), the assay is likely to be negative for infection with *M. avium* complex (MAC), which is a major source of NTM infection. This was compatible with the study's finding that IFN-γ response to both of ESAT-6 and CFP-10 was negative in all patients who were culture negative for *M. tuberculosis* and positive for MAC, except one. The latter MAC patient with a positive IFN-γ response could have coinfection with tuberculosis. On the other hand, positive reactions are expected from people infected with *M. kansasii, Mycobacterium marinum,* or *Mycobacterium szulgai* as the genes encoding both ESAT-6 and CFP-10 are present in these NTM (7). Therefore, it is not surprising that another three TB suspects positive for *M. kansasii* responded to ESAT-6 and/or CFP-10 in the whole blood IFN-γ assay.

It remains to be confirmed whether the enhanced sensitivity of the whole blood IFN-γ assay over the TST, as seen for untreated patients in this study, will also be found for people with latent tuberculosis infection. However, such a possibility can be supported by reports that contacts of patients with tuberculosis, who are possibly latently infected with *M. tuberculosis*, have stronger IFN-γ responses to *M. tuberculosis* antigens than do patients with active tuberculosis (18, 19, 21–23). Further investigations on the performance of the CFP-10/ESAT-6-based whole blood IFN-γ assay in contact investigations and in other situations where *M. tuberculosis* exposure can be quantified are required to further estimate the test performance for detecting latent tuberculosis infection.

Screening for latent tuberculosis infection is most effective if those with positive test results are likely to progress to clinical disease. A preliminary study by Doherty and coworkers (24) demonstrated a close relationship between IFN-γ responses and subsequent development of clinical tuberculosis disease in household tuberculosis contacts in Ethiopia, but this needs corroboration in

TABLE 3. CFP-10 AND ESAT-6 IFN-γ ASSAY AND MANTOUX TUBERCULIN SKIN TEST RESULTS, STRATIFIED BY AGE, FOR 110 PATIENTS WITH CULTURE-POSITIVE TUBERCULOSIS

Age (*yr*)	No. IFN-γ–tested	No. IFN-γ–positive	Percent IFN-γ–positive	No. Mantoux-tested	No. Mantoux-positive	Percent Mantoux-positive
13–30	19	17	89.5	9	9	100.0
31–40	14	14	100.0	12	7	58.3
41–50	16	15	93.8	12	9	75.0
51–60	19	19	100.0	10	5	50.0
61–70	19	17	89.5	12	9	75.0
71–80	13	12	92.3	11	6	54.5
> 80	10	8	80.0	6	1	16.7

Results for the Mantoux test are based on a 5-mm cutoff.

other populations of different immune status and background. In addition, although the current study indicates utility of the IFN-γ assay in screening adults for TB infection, further studies are required, including those in select patient populations such as children, people with X-ray evidence of prior tuberculosis, and those with HIV infection or other immunodeficiencies. Test utility would also be enhanced by studies determining the kinetics of IFN-γ response after infection, and the effect of antituberculosis therapy on IFN-γ test results.

Previous studies have demonstrated the potential of both ESAT-6 and CFP-10 for the specific detection of *M. tuberculosis* infection in humans (4, 5–10), although the method generally used to measure IFN-γ responses to these antigens, such as lymphocyte proliferation and IFN-γ enzyme-linked immunospot, are relatively complex and labor intensive to perform (25). Some of these studies have demonstrated that a combination of results from ESAT-6 and CFP-10 provides higher sensitivity than is seen with either antigen alone (7, 8). In addition, Vordermeier and coworkers demonstrated greater sensitivity with a cocktail of CFP-10 and ESAT-6 over either antigen alone, when used in an IFN-γ enzyme-linked immunospot assay (26), and Arend and coworkers showed that use of both antigens increased test sensitivity, as there were variations in responses to CFP-10 and ESAT-6 between individuals with different HLA-DR types (10). These data suggest that the combined use of both TB-specific antigens is warranted to increase sensitivity and our results support this conclusion.

In addition to the high diagnostic accuracy resulting from the use of *M. tuberculosis*–specific antigens, the whole blood IFN-γ assay offers many methodologic and logistic advantages, both over the TST and other laboratory methods of immunological testing. The test requires a single patient visit, does not induce boosting of subsequent test results, and can provide results within 1 day. Interreader variability is low and results are highly reproducible (27) as it is a controlled laboratory assay. Importantly, whole blood testing uses minimal labor and simple equipment, allowing large numbers of samples to be tested concurrently.

Conflict of Interest Statement: T.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; F.Y. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; E.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; N.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; S.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; G.H.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; I.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Induction of Protective Cellular Immunity against *Mycobacterium tuberculosis* by Recombinant Attenuated Self-Destructing *Listeria monocytogenes* Strains Harboring Eukaryotic Expression Plasmids for Antigen 85 Complex and MPB/MPT51

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We report here the induction of specific protective cellular immunity against *Mycobacterium tuberculosis* by the employment of vaccination with recombinant attenuated *Listeria monocytogenes* strains. We constructed self-destructing attenuated *L. monocytogenes* Δ2 strains carrying eukaryotic expression plasmids for the antigen 85 complex (Ag85A and Ag85B) and for MPB/MPT51 (mycobacterial protein secreted by *M. bovis* BCG/mycobacterial protein secreted by *M. tuberculosis*) molecules. Infection of these recombinant bacteria allowed expression of the genes in the J774A.1 murine macrophage cell line. Intraperitoneal vaccination of C57BL/6 mice with these recombinant bacteria was capable of inducing purified protein derivative-specific cellular immune responses, such as foot pad reactions, proliferative responses of splenocytes, and gamma interferon production from splenocytes, suggesting the efficacy of vaccination against mycobacterial infection by use of these recombinant *L. monocytogenes* strains. Furthermore, intravenous vaccination with recombinant bacteria carrying expression plasmids for Ag85A, Ag85B, or MPB/MPT51 in BALB/c mice elicited significant protective responses, comparable to those evoked by a live *Mycobacterium bovis* BCG vaccine. Notably, this is the first report to show that MPB/MPT51 is a major protective antigen in addition to Ag85A and Ag85B, which have been reported to be major mycobacterial protective antigens.

Tuberculosis (TB) remains an urgent public health problem worldwide, resulting in 8 million new cases and 2 million deaths each year (14). Outbreaks of TB, especially in immunocompromised people, such as aged groups and AIDS patients, have also been reported. In addition, the appearance of multidrug-resistant *Mycobacterium tuberculosis* strains is also a serious issue in the world.

The only TB vaccine currently available is the attenuated *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG), which has been reported to have a variable protective efficacy, ranging from 0 to 85% in different controlled studies (6). Therefore, there remains an urgent need for an improved vaccine. A DNA vaccine is one of the most promising candidates for future TB vaccines. Many reports on DNA vaccination against TB have been accumulating. Secreted molecules have been known to be recognized by the protective immune response against TB. In these reports, various target antigens (Ags) for TB DNA vaccination have been reported, including the Ag85 complex molecules, Hsp65, Hsp70, the 38-kDa Ag, and ESAT-6 (reviewed in reference 28).

Ag85 complex molecules have been reported to be the dominant secreted Ags expressed by nearly all mycobacterial species analyzed so far (reviewed in reference 39). The complex consists of three structurally related components, namely Ag85A (p32A; 32-kDa Ag), Ag85B (p30; 30-kDa Ag, also termed α Ag), and Ag85C. Ag85 complex molecules are cross-reactive Ags and are highly conserved among *Mycobacterium* spp. The genes encode proteins with fibronectin-binding capacities (1) and mycolyltransferase activities, which are involved in the final stage of mycobacterial cell wall assembly (5). Ag85A and Ag85B have been reported to stimulate B- and T-cell responses in TB patients (24, 25), and immunization with Ag85A and Ag85B proteins induced protection against an aerosol challenge with *M. tuberculosis* in mice and guinea pigs, respectively (19). In addition, reports of successful naked DNA vaccines against TB, employing the Ag85A (3, 4, 9, 13, 21, 29, 36, 37) and Ag85B (22, 29, 37) genes, have also accumulated. According to these reports, the Ag85A and Ag85B molecules seem to be two of the most promising candidates for future subunit TB vaccines. Another molecule, MPB/MPT51 (mycobacterial protein secreted by *M. bovis* BCG/mycobacterial protein secreted by *M. tuberculosis*), has also been reported to be related to this family (31). The amino acid sequence deduced for MPB51 (GenBank/EMBL/DDBJ accession number D26486) is identical to the sequence deduced for MPT51 of *M. tuberculosis* strains H37Rv (AL022076) and CDC1551 (AE007185). So far, MPB/MPT51 has not been reported as a target Ag for vaccination against *M. tuberculosis*.

DNA vaccines offer many advantages over other methods

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of immunization: they have a relatively easy design and construction by recombinant DNA techniques, a strong induction of cellular immunity, chemical stability, a relatively low cost, and so on (reviewed in references 2 and 12). For successful results with DNA vaccination (for example, in the case of Ag85A), however, intramuscular immunization with large amounts (50 to 100 μg) of plasmid DNA was reported to be necessary (36), and the induction of immunity with intramuscular immunization of plasmid DNA is poor in terms of reproducibility (41). Recently, several investigators used attenuated intracellular bacteria as the carriers of DNA vaccines (reviewed in reference 11). These bacterial carrier systems have several special features, including direct delivery of the plasmid DNA to professional antigen-presenting cells and the possibility of oral administration. Bacteria utilized as this type of vaccine carrier include *Salmonella* (8) and *Shigella* (35) as well as *Listeria* (10). Gram-negative carriers such as *Salmonella* and *Shigella* have the disadvantage of containing abundant amounts of toxic lipopolysaccharide. Therefore, *Listeria monocytogenes*, a gram-positive bacterium, is a good candidate for a carrier. Furthermore, this bacterium is considered a possible effective recombinant vaccine vector based on its predilection for professional antigen-presenting cells such as macrophages and dendritic cells and its capacity to escape from phagolysosomes and to live in the cytoplasm of host cells (34, 38). In addition, this bacterium has been reported to have the ability to induce T-helper cell type 1 (Th1) immune responses (20). These features are favorable for eliciting effective cellular immunity against TB. Dietrich et al. (10) reported a DNA vaccination system using an attenuated self-destructing *L. monocytogenes* strain. They demonstrated the feasibility of the system in a cell culture system. They used a deletion mutant of *L. monocytogenes* Δ2 that lacks the entire lecithinase operon, including the virulence-associated genes *actA*, *mpl*, and *plcB* (17). This strain can infect macrophages and replicate in the cytoplasm but cannot spread to adjacent cells. This attenuated mutant was introduced with a plasmid containing the gene for lysis protein PLY118 of the listerial bacteriophage A118. PLY118 expression was controlled by the *actA* promoter, which is active when *L. monocytogenes* is in the host cell cytoplasm. Thus, this *L. monocytogenes* mutant escapes from the phagosome and then lyses when the PLY118 gene is expressed in the cytoplasm. Autolysis of the *L. monocytogenes* mutant apparently releases the plasmid DNA into the host cell cytoplasm, allowing expression of the transgene in the host cells. However, it was still unknown whether this DNA vaccine carrier system is capable of inducing specific immunity and protective immunity against infection in vivo.

For this study, we examined the inducibility of protective cellular immunity against *M. tuberculosis* by immunization of mice with this attenuated *L. monocytogenes* strain carrying a eukaryotic expression plasmid for Ag85A, Ag85B, or MPB51. The results showed that vaccination with the attenuated self-destructing *L. monocytogenes* strain could induce protective cellular immunity against *M. tuberculosis* infection. Furthermore, we show for the first time that MPB/MPT51, which is related to Ag85 family molecules, is a major protective Ag.

MATERIALS AND METHODS

Bacteria and plasmids. *M. bovis* BCG (substrain Tokyo) was purchased from Japan BCG Inc. (Tokyo, Japan). The attenuated *L. monocytogenes* strain Δ2 (10, 17) and plasmids p3LOVA118 and pcDNA3L (10) were kindly provided by Werner Goebel, Guido Dietrich, and Ivaylo Gentschev (University of Würzburg, Germany). Attenuated *L. monocytogenes* Δ2 was cultured in brain heart infusion (BHI) broth (Becton Dickinson, Sparks, Md.) at 37°C. *Escherichia coli* DH5α was cultured in L broth. *M. tuberculosis* H37Rv was kindly donated by Isamu Sugawara (Research Institute of Tuberculosis, Tokyo, Japan).

Construction of recombinant plasmids p3L118R-Ag85A, p3L118R-Ag85B, and p3L118R-MPB51. The NruI-NotI fragment of p3LOVA118, covering half of the cytomegalovirus (CMV) promoter and the ovalbumin epitope region, was removed and replaced with the corresponding region of pcDNA3L, resulting in p3L118R. This procedure removed the ovalbumin epitope region from p3LOVA118 and recreated a NotI site for future subcloning of genes of interest under control of the CMV promoter. The BCG Ag85A, Ag85B, and MPB51 genes were amplified from plasmids pMB49 (for Ag85A and MPB51) (31) and pαL-1 (30) (for Ag85B) by PCRs with the following primer pairs: 5′-ATAAGA ATGCGGCCGCACCATGCAGCTTGTTGACAGG-3′ and 5′-ATAGTTTAG CGGCCGCTGTTCGGAGCTAGGCGC-3′ for Ag85A, 5′-ATAAGAATGCG GCCGCACCATGACAGACGTGAGCCGA-3′ and 5′-ATAGTTTAGCGGCC GCGGGCCCGTTGATCCCGTCAGCCGGC-3′ for Ag85B, and 5′-ATAAGA ATGCGGCCGCTCGAGCACCATGAAGGGTCGGTCGGCG-3′ and 5′-AT AGTTTAGCGGCCGCGGGCCCGGCACCTGGCTTAGCGGA-3′ for MPB51 (underlined text indicates a NotI site). These PCR fragments were digested with NotI and inserted into a NotI site of p3L118R. The integrity of the nucleotide sequences was validated by automated DNA sequencing (ABI PRISM 310 genetic analyzer; Applied Biosystems, Foster City, Calif.) using a dye primer cycle sequencing kit (Applied Biosystems). The resultant plasmids were introduced into the attenuated *L. monocytogenes* Δ2 strain by electroporation, as described below.

Electroporation of plasmids into *L. monocytogenes* Δ2. The electroporation procedure was basically in accordance with a previously described protocol (33). Briefly, *L. monocytogenes* Δ2 cells were shaken in 200 ml of BHI broth at 37°C until an optical density at 600 nm of 1.0. Next, 2,000 U of penicillin G was added and the culture was subjected to a 1-h incubation. The cells were harvested, washed twice with sucrose electroporation buffer (1 mM HEPES [pH 7.0], 0.5 M sucrose), and resuspended in 500 μl of the buffer. One hundred microliters of the cell suspension and 1 μg of one of the expression plasmids were then transferred to an electroporation cuvette and subjected to electroshock with a Gene-Pulser electroporation apparatus (Bio-Rad Laboratories, Hercules, Calif.). The electroporation conditions were as follows: cuvette gap, 0.4 cm; voltage, 2.5 kV; field strength, 6.25 kV/cm; capacitor, 25 μF; and resistor, 200 Ω. Next, the cell solution was incubated on ice for 10 min, added to 0.7 ml of BHI broth, and incubated at 37°C for 1 h. After centrifugation at 1,200 × *g* for 15 min at 4°C, 0.6 ml of the solution was removed. The remaining solution was plated onto a Trypticase soy agar plate (Becton Dickinson) containing 12.5 μg of tetracycline/ml and was incubated at 37°C for 18 h. Resultant tetracycline-resistant colonies were cultured and stored. These were named Δ2/p3L118R, Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, and Δ2/p3L118R-MPB51 and harbored the recombinant plasmids p3L118R, p3L118R-Ag85A, p3L118R-Ag85B, and p3L118R-MPB51, respectively.

Mammalian cell culture. The murine macrophage-like cell line J774A.1 (American Type Culture Collection, Manassas, Va.) and spleen cells of immunized mice were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 in an incubator.

Infection of J774A.1 cells with recombinant *L. monocytogenes* Δ2 strains. J774A.1 cells (5×10^5 cells) were plated on 60-mm-diameter plates at the beginning of experiments. The medium was renewed 24 h before the experiments. Recombinant Δ2 strains (10^5 cells) were added to J774A.1 cells. After 5 h, 10 μg of gentamicin sulfate/ml was added to the medium to remove extracellular bacteria. After a 36-h incubation, the infected cells were harvested.

Reverse transcription (RT)-PCR analysis for Ag85A, Ag85B, or MPB51 gene detection. Δ2/p3L118R-, Δ2/p3L118R-Ag85A-, Δ2/p3L118R-Ag85B-, or Δ2/p3L118R-MPB51-infected J774A.1 cells were harvested, and total RNAs were prepared from the cells by use of Isogen RNA extraction solution (Nippon Gene, Tokyo, Japan). Single-stranded cDNAs were synthesized with Molony murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, Md.) and then were used for PCR analysis. The primers used for Ag85A, Ag85B, and MPB51 gene detection were as follows: for Ag85A, 5′-AGGCCAACAGGCAC GTCAA-3′ and 5′-ACATGTCGGAGGCCTTGTA-3′; for Ag85B, 5′-GAACA ACTCACCTGCGGTT-3′ and 5′-CATCGACAAGCCGATTGC-3′; and for

MPB51, 5′-GATGTCAGTAACTGGGTCAC-3′ and 5′-ACATTCCGTTGGTG TCCACA-3′. To refute the possibility of contamination of the plasmids in cDNA pools, we performed PCRs with combinations of a primer located in the CMV enhancer-promoter region of p3L118R, 5′-GGTGGGAGGTCTATATAAGC-3′, and the reverse primers for the Ag85A, Ag85B, and MPB51 genes.

Mice. C57BL/6 and BALB/c mice (Japan SLC, Hamamatsu, Japan) were kept under specific-pathogen-free conditions and fed autoclaved food and water ad libitum at the Experimental Animal Institute of the Hamamatsu University School of Medicine. All animal experiments were performed according to the Guidelines for Animal Experimentation, Hamamatsu University School of Medicine.

Immunization procedures. Mice were immunized intraperitoneally (i.p.) (C57BL/6; $\sim 10^7$ CFU) or intravenously (i.v.) (BALB/c; $\sim 10^6$ CFU) with a recombinant attenuated *L. monocytogenes* Δ2 strain three times at 2-week intervals. As a control, mice were also immunized with BCG i.p. once (C57BL/6, $\sim 10^7$ CFU) or subcutaneously twice at a 2-week interval (BALB/c, $\sim 10^6$ CFU).

Genomic DNA PCR. Δ2/p3L118R-MPB51 or Δ2/p3L118R *Listeria* ($\sim 10^8$ CFU) was injected i.p. into C57BL/6 mice, and Δ2/p3L118R-MPB51 or *L. monocytogenes* EGD (a parental strain of Δ2; $\sim 10^7$ CFU) was injected i.v. into BALB/c mice. One day after the injection, tissue cell suspensions from injected mice were prepared and washed three times after the lysis of erythrocytes with Tris-buffered 0.83% ammonium chloride solution. After a brief centrifugation, the cells were added to 10 volumes of proteinase K solution (1 mg/ml; Boehringer Mannheim GmbH, Mannheim, Germany) in 10 mM Tris (pH 7.4), 10 mM EDTA, 150 mM NaCl, and 0.4% sodium dodecyl sulfate and were incubated for 15 min at 65°C. The cells were further incubated in the same solution overnight at 37°C. Genomic DNA was prepared from the cells after phenol extraction and ethanol precipitation. A nested PCR was performed with 1 μg of genomic DNA for MPB51 gene detection. The first-round PCR was performed with the same primer pairs that were used for RT-PCR analysis, and the second-round PCR was performed with 1 μl of the first-round PCR solution (20-μl total volume) and the following primer pairs located just inside of the first-round PCR primers: 5′-CGCGGGTAACGCGATGAACAC-3′ and 5′-CACACCGCCGAATTGCT GCAT-3′. For both PCRs, the conditions were 25 cycles of 94°C for 30 s, 62°C for 50 s, and 72°C for 30 s. The expected size of the MPB51 PCR product was 341 bp.

Delayed-type hypersensitivity (DTH) reaction. Purified protein derivative (PPD) was purchased from Japan BCG Inc. C57BL/6 mice were injected with 5 μg of PPD in 50 μl of phosphate-buffered saline (PBS) in the left hind foot pad. As controls, mice were injected with 50 μl of PBS alone in the right hind foot pad. The swelling of foot pads was measured with a caliper meter (Mitsutoyo Corp., Osaka, Japan) 48 h after injection. Naïve mice were treated in the same way as the controls for nonspecific swelling.

Lymphocyte proliferation assay. Spleen cells (10^5/well) from the immunized C57BL/6 mice were incubated for 48 h at 37°C in 96-well round-bottom tissue culture plates in the presence or absence of 5 μg of PPD/ml. After 48 h of culturing, de novo DNA synthesis was assessed by the addition of 0.5 μCi of [methyl-^{3}H]thymidine (10 Ci/mmol; ICN Biochemicals, Irvine, Calif.)/well for the last 12 h of culture. Quintuplicate cultures were harvested onto glass fiber filters, and the radioactivity was counted by liquid scintillation. The [methyl-^{3}H]thymidine incorporation was calculated in counts per minute per 10^4 cells.

Cytokine ELISA. Spleen cells were harvested from the immunized C57BL/6 mice. Recovered cells were incubated for 4 days in 24-well plates at 2×10^6 cells/well in RPMI–10% fetal bovine serum in the presence or absence of 5 μg of PPD solution/ml. Concentrations of gamma interferon (IFN-γ), interleukin-4 (IL-4), and IL-5 in the culture supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA) as described elsewhere (40). For the sandwich ELISA, the following combinations of coating and biotinylated monoclonal antibodies were used: R4-6A2 and XMG1.2 for IFN-γ, 11B11 and BVD6-24G2 for IL-4, and TRFK5 and TRFK4 for IL-5. All monoclonal antibodies were purchased from BD PharMingen (San Diego, Calif.). The amounts of cytokines were calculated by using standard murine recombinant cytokine curves run on the same immunoplate.

Semiquantitative RT-PCR for IFN-γ gene. Immune spleen cells (C57BL/6 mice) were cultured for 48 h at 10^7 cells/ml in the presence or absence of 5 μg of PPD solution/ml. Total RNAs were extracted from cells by use of Isogen RNA extraction solution (Nippon Gene). Single-stranded cDNAs were synthesized with Molony murine leukemia virus reverse transcriptase (Life Technologies) and then used in PCRs for IFN-γ gene detection as described elsewhere (40).

In vivo protection assay. Immunized BALB/c mice were infected with 5×10^5 CFU of *M. tuberculosis* H37Rv i.v. 2 months after the last immunization. Mice were sacrificed 10 weeks later, and the bacterial numbers in the spleens, livers, and lungs were counted in CFU on Middlebrook 7H11 medium (Becton Dickinson).

Statistics. Data from multiple experiments were expressed as means ± standard deviations (SD). Statistical analyses were performed with the StatView-J 4.02 statistics program (Abacus Concepts, Berkeley, Calif.). Data were analyzed by Fisher's protected least significant difference.

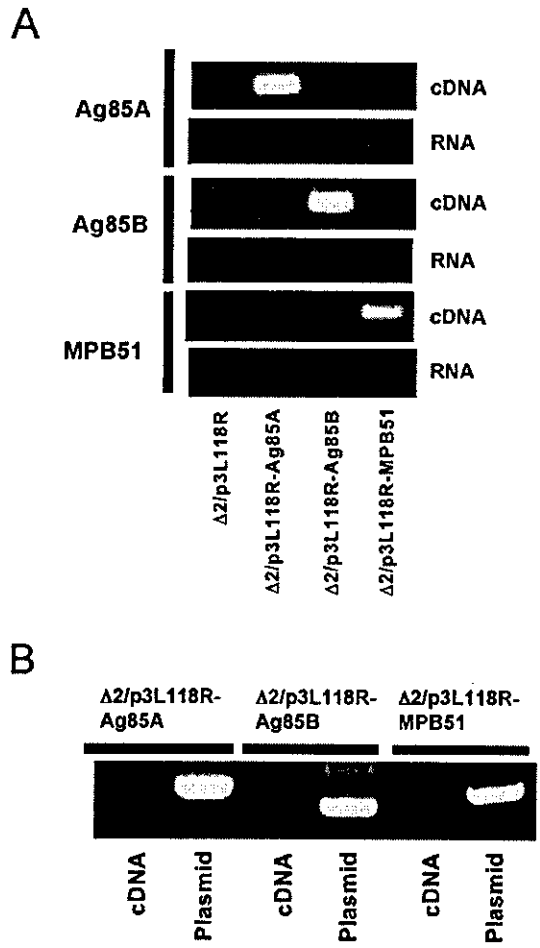

FIG. 1. Expression of Ag85A, Ag85B, and MPB51 mRNAs in murine macrophage cells infected with Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51. (A) Murine macrophage cell line J774A.1 was infected with Δ2/p3L118R, Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51. Total RNAs from these infected cells were recovered and reverse transcribed with random hexamers to make cDNA pools. PCRs were then performed with Ag85A, Ag85B, and MPB51 gene-specific primers (cDNA panels). After the recovery of total RNAs, the same PCRs were performed for Ag85A, Ag85B, or MPB51 gene detection without RT (RNA panels). (B) To refute the possibility of contamination of p3L118R-Ag85A, p3L118R-Ag85B, or p3L118R-MPB51 in the cDNA pools used for panel A, we subjected the cDNA pools to PCRs with primer sets by which only the plasmid DNAs, not the transcripts, were detected. See Materials and Methods for details.

RESULTS

Infection of recombinant *L. monocytogenes* allowed expression of genes in J774A.1 murine macrophage cell line. J774A.1 murine macrophage-like cells were infected with an *L. monocytogenes* Δ2 mutant carrying the plasmid p3L118R, p3L118R-

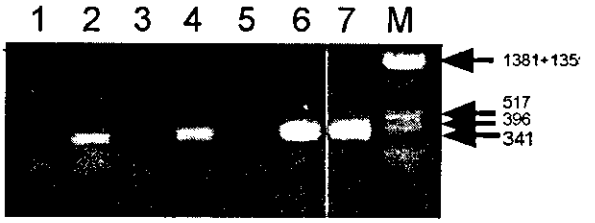

FIG. 2. Detection of p3L118R-MPB51 plasmid in tissues of Δ2/p3L118R-MPB51-injected mice. Mice were injected with Δ2/p3L118R-MPB51 or control *Listeria* i.p. (C57BL/6) or i.v. (BALB/c). Genomic DNA was prepared from tissues of the injected mice 1 day after injection, and a nested PCR was performed for MPB51 DNA detection. Lane 1, spleen of C57BL/6 mouse injected with Δ2/p3L118R control; lane 2, spleen of C57BL/6 mouse injected with Δ2/p3L118R-MPB51; lane 3, spleen of BALB/c mouse injected with *L. monocytogenes* EGD; lane 4, spleen of BALB/c mouse injected with Δ2/p3L118R-MPB51; lane 5, liver of BALB/c mouse injected with *L. monocytogenes* EGD; lane 6, liver of BALB/c mouse injected with Δ2/p3L118R-MPB51; lane 7, control p3L118R-MPB51 plasmid. A size marker was also loaded (lane M). DNA fragment sizes are shown to the right.

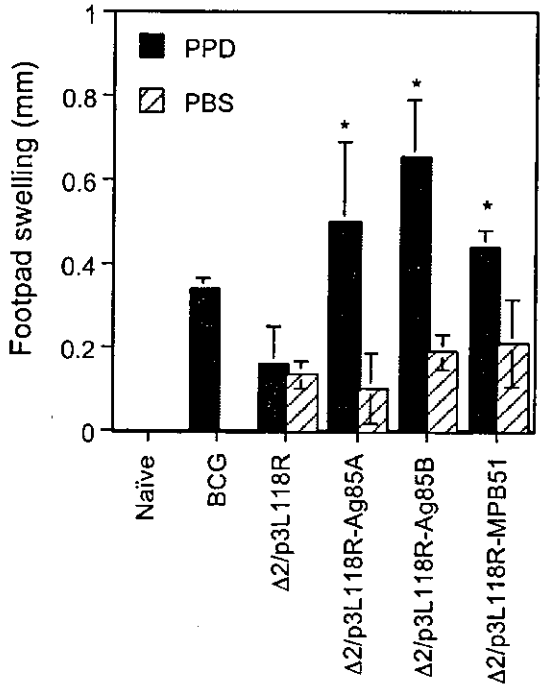

FIG. 3. Foot pad swelling responses of mice immunized with Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51. C57BL/6 mice were immunized with Δ2/p3L118R, Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51 three times at 2-week intervals. The data for mice immunized once with *M. bovis* BCG are also shown as a control. One month after the last immunization, foot pad swelling responses directed against PPD were examined. Black bars, footpad swelling after in vivo PPD stimulation; hatched bars, foot pad swelling with PBS alone. The means ± SD of four to five mice per group are shown. Asterisks indicate statistically significant ($P \leq 0.001$) differences with the value for a control (Δ2/p3L118R) immunization.

Ag85A, p3L118R-Ag85B, or p3L118R-MPB51. Thirty-six hours after infection, the infected cells were harvested for the isolation of total RNA. RT-PCR was then performed to confirm the expression of Ag85A, Ag85B, or MPB51 mRNA in the cells. As shown in Fig. 1A, clear bands for these mRNAs were detected after RT of total RNA solutions but not before reverse transcriptase treatment. In addition, to refute the possibility of contamination of plasmids p3L118R-Ag85A, p3L118R-Ag85B, and p3L118R-MPB51 in the cDNA pools used, we subjected the cDNA pools to PCRs with relevant primer sets, by which only the plasmid DNAs, but not the transcripts, were detected (see Materials and Methods for details). The results showed that no bands were detected with the cDNA pools by PCR, while p3L118R-Ag85A, p3L118R-Ag85B, and p3L118R-MPB51 controls gave specific bands, indicating no contamination of plasmids in the cDNA pools. These data indicate that the Ag85A, Ag85B, and MPB51 genes were expressed in J774A.1 cells by this attenuated *L. monocytogenes* system.

Detection of injected plasmid DNA in tissues of mice infected with recombinant attenuated *L. monocytogenes*. Δ2/p3L118R-MPB51 recombinant *Listeria* was injected i.p. (C57BL/6 mice) or i.v. (BALB/c mice). In order to check for p3L118R-MPB51 plasmid transfer to tissues of the injected mice, we prepared genomic DNAs from cells of the tissues and performed PCR analysis for MPB51 gene detection. As shown in Fig. 2, we observed an MPB51-specific band only for DNAs derived from mice injected with Δ2/p3L118R-MPB51 *Listeria*. The PCR was performed with tissue cells washed with PBS, suggesting that the p3L118R-MPB51 plasmid was transferred into host cells after recombinant *Listeria* injection. It is noteworthy that we observed no colonies of carrier *L. monocytogenes* in the spleens of the i.p. immunized C57BL/6 mice by plating of the tissue homogenates on Trypticase soy agar (data not shown).

PPD-specific DTH reaction with recombinant attenuated *L. monocytogenes* vaccination. For effective protective immunity against *M. tuberculosis*, specific cellular immunity against the bacterium plays a critical role. We first examined DTH responses of C57BL/6 mice immunized with Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51. As shown in Fig. 3, mice immunized with these recombinant *Listeria* strains significantly responded to PPD, but not to PBS alone. Similar, but lower, responses were obtained for mice immunized with *M. bovis* BCG. Mice immunized with the Δ2/p3L118R control strain failed to show specific DTH reactions to PPD. These results indicate that mice immunized with Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51 successfully elicited cellular immunity against *M. tuberculosis*.

PPD-specific lymphocyte proliferation after recombinant attenuated *L. monocytogenes* vaccination. We next examined proliferative responses of splenocytes derived from the immunized C57BL/6 mice in response to in vitro PPD stimulation. As shown in Fig. 4, a strong proliferative response was observed in control BCG-immunized mice. Immunization with recombinant Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51 also caused significant proliferative responses, but the levels of specific proliferation were lower than that evoked by immunization with *M. bovis* BCG. Splenocytes of mice immunized with the Δ2/p3L118R control recombinant *L. monocytogenes* strain did not have a significant response.

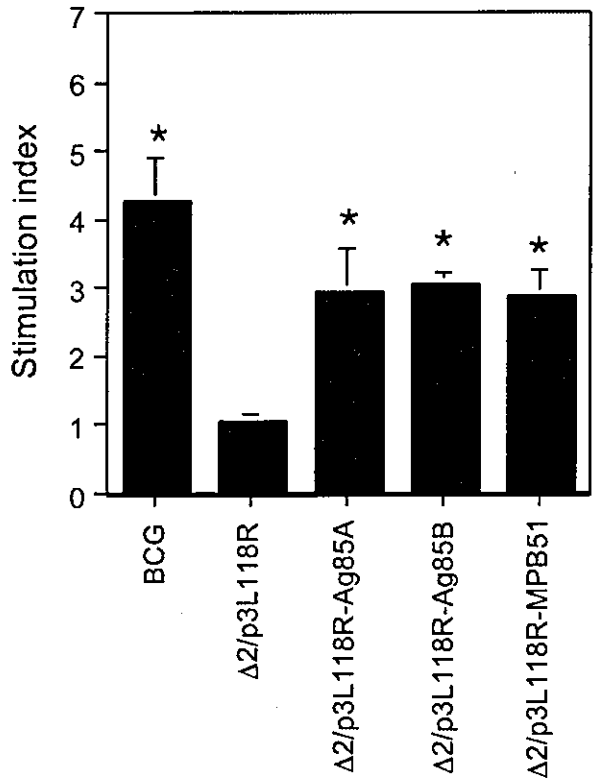

FIG. 4. PPD-specific splenocyte proliferation of mice immunized with Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51. C57BL/6 mice were immunized with Δ2/p3L118R, Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51 three times at 2-week intervals. The data for mice immunized once with *M. bovis* BCG are also shown as a control. Spleen cells from the immunized mice were harvested 1 month after the last immunization, cultured in vitro in the presence or absence of 5 μg of PPD/ml for 48 h, and pulsed with 0.5 μCi of [methyl-^{3}H]thymidine/ml for the last 12 h. The values represent stimulation indexes (the values after in vitro stimulation in the presence of PPD divided by the values in the absence of PPD). The means ± SD of quintuplicate determinations from a representative experiment of three independent experiments are shown. Asterisks indicate statistically significant ($P < 0.0001$) differences with the value for a control (Δ2/p3L118R) immunization.

PPD-specific cytokine production with recombinant attenuated *L. monocytogenes* vaccination. IFN-γ is known to be a key factor for the elicitation of effective protection against *M. tuberculosis*. Therefore, employing RT-PCR analysis, we semiquantitatively assessed IFN-γ mRNA expression from splenocytes of immunized C57BL/6 mice upon PPD stimulation. As shown in Fig. 5, IFN-γ mRNA-specific bands were clearly detected in PPD-stimulated splenocytes of C57BL/6 mice immunized with recombinant *Listeria* strain Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51. The strengths of the bands were comparable to that of mice immunized with *M. bovis* BCG. Again, splenocytes of mice immunized with the Δ2/p3L118R control gave only a faint IFN-γ mRNA-specific band.

In addition, we examined the cytokine production of splenocytes from immunized C57BL/6 mice by a sandwich ELISA for IFN-γ, IL-4, and IL-5 (Table 1). Correlating with the results of

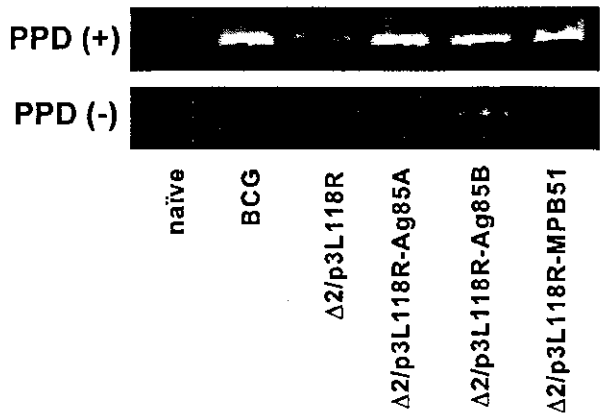

FIG. 5. IFN-γ mRNA expression by spleen cells of mice immunized with Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51. C57BL/6 mice were immunized with Δ2/p3L118R, Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51 three times at 2-week intervals. The data for mice immunized once with *M. bovis* BCG are also shown as a control. Spleen cells from the immunized mice were harvested 1 month after the last immunization and cultured in vitro in the presence [PPD (+)] or absence [PPD (−)] of 5 μg of PPD/ml for 48 h. IFN-γ mRNA expression was evaluated by semiquantitative RT-PCR with IFN-γ-specific primers.

RT-PCRs, splenocytes from mice immunized with *M. bovis* BCG or a recombinant *Listeria* strain harboring the Ag85A, Ag85B, or MPB51 gene produced high amounts of IFN-γ after in vitro stimulation with PPD. We observed the production of moderate levels of IFN-γ from spleen cells of naïve mice and control *Listeria* (Δ2/p3L118R)-immunized mice upon PPD stimulation. We did not detect significantly enhanced production of IL-4 or IL-5 for any of the mice examined.

Recombinant attenuated *L. monocytogenes* vaccination conferred protective immunity against *M. tuberculosis* infection comparable to *M. bovis* BCG immunization in BALB/c mice.

TABLE 1. Cytokine production by spleen cells from mice immunized with Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51

Mouse group	Stimulation with PPD[a]	Cytokine production (pg/ml)[b]		
		IFN-γ	IL-4	IL-5
Naïve	−	131	122	71
	+	868	54	0
BCG immunized	−	186	92	52
	+	4,103	70	6
Δ2/p3L118R immunized	−	51	44	10
	+	740	94	31
Δ2/p3L118R-Ag85A immunized	−	218	74	43
	+	1,728	35	0
Δ2/p3L118R-Ag85B immunized	−	210	44	18
	+	3,432	66	14
Δ2/p3L118R-MPB51 immunized	−	191	19	0
	+	4,093	31	0

[a] Spleen cells from immunized C57BL/6 mice (2×10^6 cells per well) were cultured in the presence (+) or absence (−) of 5 μg of PPD/ml.

[b] After 4 days, cytokine concentrations in culture supernatants were quantified by IFN-γ-, IL-4-, and IL-5-specific ELISA, as described in Materials and Methods. The values for naïve and BCG-immunized mice are also shown as controls. Averages of duplicate representative data from several similar experiments are shown.

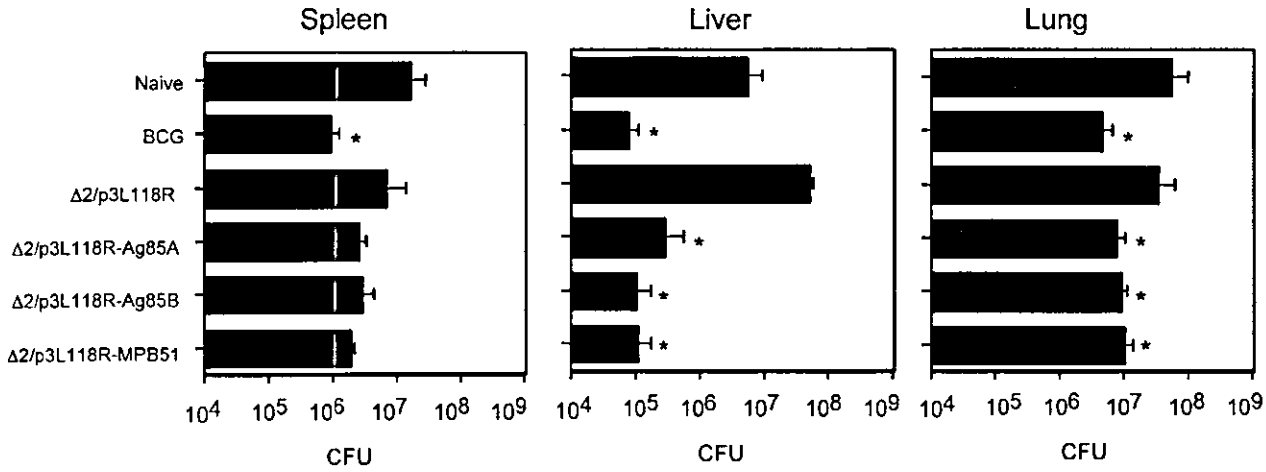

FIG. 6. In vivo protective activity of mice immunized with Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51 against virulent *M. tuberculosis*. BALB/c mice were immunized with Δ2/p3L118R, Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51 three times at 2-week intervals. The data for mice immunized once with *M. bovis* BCG are also shown as a control. The mice were challenged i.v. with live *M. tuberculosis* H37Rv. Numbers of CFU in the spleens, livers, and lungs were determined 10 weeks later. The means ± SD of six mice are shown. Asterisks indicate statistically significant ($P < 0.05$) differences with the value for a control (Δ2/p3L118R) immunization.

We evaluated the effects of recombinant attenuated *Listeria* vaccination on protective immunity against *M. tuberculosis* H37Rv infection and compared them with those of *M. bovis* BCG vaccination. At first, we used C57BL/6 mice for the experiments, but the relative resistance against *M. tuberculosis* infection of the strain hampered the evaluation of the vaccination effects. Therefore, we used BALB/c mice for the evaluation. Ten weeks after i.v. injection with *M. tuberculosis* H37Rv, spleens, livers, and lungs were prepared from the immunized mice and the numbers of CFU of *M. tuberculosis* H37Rv in these tissues were evaluated. Figure 6 shows viable colony counts for tissues from mice immunized with Δ2/p3L118R-Ag85A, Δ2/p3L118R-Ag85B, or Δ2/p3L118R-MPB51 *Listeria* compared with those from naïve mice, mice immunized with the Δ2/p3L118R control, and BCG-vaccinated mice. The protective effects of these recombinant *Listeria* immunizations were obvious in all tissues examined and were comparable to those of live BCG vaccination. In the liver, particularly, we detected an approximately 2-orders-of-magnitude reduction in CFU for Ag85A, Ag85B, and MPB51 DNA vaccine- and live BCG-immunized mice.

DISCUSSION

From the findings described in this paper, we drew the following conclusions concerning the attenuated self-destructing *L. monocytogenes*-harboring DNA vaccine against *M. tuberculosis*. (i) Inoculation with recombinant *L. monocytogenes*-harboring plasmid DNA vaccines for Ag85 complex and MPB/MPT51 molecules is able to induce specific type 1 cellular immune responses in spleen cells of mice. (ii) Inoculation with these vaccines can confer protective immunity against TB. (iii) The MPB/MPT51 molecule, which is related to the Ag85 family, appears to be a major protective Ag, in addition to Ag85A and Ag85B.

We detected a significant level of PPD-specific IFN-γ secretion, which is a hallmark of type 1 immune responses and is considered an important factor in the protective immunity against *M. tuberculosis* (7, 15, 23), in splenocytes of mice immunized with attenuated recombinant *Listeria* harboring an Ag85A, Ag85B, or MPB/MPT51 DNA vaccine (Table 1). The production of moderate levels of IFN-γ from splenocytes of naïve mice and control *Listeria* (Δ2/p3L118R)-immunized mice may be caused by nonspecific responses of these mice against PPD.

Cellular immunity, including $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ Th1 cells, has been reported to play critical roles in effective protective immunity against *M. tuberculosis* (reviewed in references 16 and 32). In this context, the attenuated *Listeria* immunization system shown here should be a favorable immunization method, as it is able to elicit effective type 1 cellular immune responses against *M. tuberculosis*. Furthermore, an attenuated *Listeria* strain harboring the suicide gene *ply118* was revealed to be almost nontoxic, since inoculation with ~10^8 CFU of the attenuated *Listeria*, but not virulent *L. monocytogenes*, failed to kill even IFN-γ receptor knockout mice as well as C57BL/6 wild-type mice (data not shown). Also, we could not detect carrier *L. monocytogenes*, although the plasmid DNA vaccines were detected in the spleens of i.p. immunized C57BL/6 mice (data not shown).

Several heterologous carrier systems for mycobacterial Ags have been reported. Zhu et al. (42) showed that the recombinant vaccinia virus system for *M. tuberculosis*-derived 19- and 38-kDa glycolipoproteins is effective for protection against murine *M. tuberculosis* infection. Hess et al. (18) reported that a recombinant *Salmonella enterica* serovar Typhimurium vaccine which secretes Ag85B is effective for the induction of pathogen-specific IFN-γ and tumor necrosis factor and also for protection against murine TB. It will be interesting to compare the system shown here with these systems in terms of the induction of protective immunity against *M. tuberculosis*.

As a general rule, the determination of a target Ag is very important for the development of effective DNA vaccines

against bacterial infection. Many reports have already shown the effectiveness of Ag85A and Ag85B for eliciting protective immunity against *M. tuberculosis*. We also confirmed with our system that Ag85A and Ag85B are capable of inducing cellular and protective immunity. In addition, we evaluated the effectiveness of MPB/MPT51 as a target Ag for an anti-TB vaccine. Our results indicate that MPB/MPT51 is also a protective Ag and is comparable to Ag85A and Ag85B. In particular, immunization with Δ2/p3L118R-MPB51 induced enhanced PPD-specific IFN-γ production from splenocytes, the expression level of which was comparable to that by BCG immunization. So far, MPB/MPT51 has not been reported as a target Ag for vaccination. Therefore, it is interesting and important to examine the antigenicity of the molecule in detail to study, for example, the capacity to induce specific $CD4^+$- and $CD8^+$-T-cell effectors, and to identify the T-cell epitopes in the molecule. We identified T-cell epitopes in C57BL/6 and BALB/c mice (M. Suzuki, T. Aoshi, T. Nagata, and Y. Koide, submitted for publication). The spleen cells derived from Δ2/p3L118R-MPB51-immunized mice were able to induce IFN-γ in response to these epitope peptides, indicating that the responses are MPB51 specific (data not shown).

For the induction of effective immunity, the route of vaccination is an important factor. The *Listeria* carrier system is suitable for the induction of mucosal immunity. Particularly, intranasal inoculation of our recombinant *Listeria* strains may be capable of inducing protective T-cell immune responses in the lung. That study is now in progress. For the present study, however, we immunized mice with *Listeria* i.p. (C57BL/6 mice) or i.v. (BALB/c mice). The main reason for not choosing the oral route for immunization is that the mouse was reported not to be a good model for the entry of *L. monocytogenes* into intestinal epithelium due to a Glu-to-Pro substitution in mouse E-cadherin, which serves as a receptor for internalin A of *L. monocytogenes* (26, 27). In humans, however, oral immunization with *L. monocytogenes*-harboring plasmid DNA vaccines seems to be a possible choice for DNA vaccine delivery. Although mice are devoid of the E-cadherin molecule, *L. monocytogenes* may have a capacity to enter into M cells located in the intestinal epithelium. Therefore, oral administration of attenuated *L. monocytogenes* strains is also worthwhile to try in mice.

Taken together, we show here that DNA vaccines with the attenuated self-destructing *L. monocytogenes* carrier system may be favorable DNA vaccination systems in vivo when accompanied with the adjuvanticity to induce Th1-type immune responses and the predilection of the bacterium to interact with macrophages.

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The development of vaccines against SARS corona virus in mice and SCID-PBL/hu mice

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Abstract

We have investigated to develop novel vaccines against SARS CoV using cDNA constructs encoding the structural antigen; spike protein (S), membrane protein (M), envelope protein (E), or nucleocapsid (N) protein, derived from SARS CoV. Mice vaccinated with SARS-N or -M DNA using pcDNA 3.1(+) plasmid vector showed T cell immune responses (CTL induction and proliferation) against N or M protein, respectively. CTL responses were also detected to SARS DNA-transfected type II alveolar epithelial cells (T7 cell clone), which are thought to be initial target cells for SARS virus infection in human. To determine whether these DNA vaccines could induce T cell immune responses in humans as well as in mice, SCID-PBL/hu mice was immunized with these DNA vaccines. As expected, virus-specific CTL responses and T cell proliferation were induced from human T cells. SARS-N and SARS-M DNA vaccines and SCID-PBL/hu mouse model will be important in the development of protective vaccines.

Keywords: SARS DNA vaccine; SCID-PBL/hu; Human CTL

1. Introduction

The causative agent of severe acute respiratory syndrome (SARS) has been identified as a new type of corona virus, SARS corona virus (SARS CoV) [1–3]. SARS has infected more than 8400 patients in about 7 months in over 30 countries and caused more than 800 deaths. The deadly epidemic has had significant impacts on many health, social, economic and political aspects. SARS is assumed to resurge in the near future. However, no SARS vaccine is currently available for clinical use. Therefore, we have developed novel vaccine can-

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didates against SARS CoV using cDNA constructs encoding the structural antigens; S, M, E, or N protein. In immunized mice, neutralizing antibodies against the virus and T cell immunity against virus-infected-cells were studied, since these immunities play important roles in protection against many virus infections. In particular, $CD8^+$ CTL plays an important role in T cell immunity dependent protection against virus infections and the eradication of murine and human cancers [4,5]. In the present study, a type II alveolar epithelial cell clone, T7, was used for analyzing precise mechanism of CTL against SARS CoV membrane antigens, as the SARS-CoV infects alveolar epithelial cell in the lungs [6]. Furthermore, the SCID-PBL/hu model, which is capable of analyzing in vivo human immune response, was also used because it is a more relevant translational model for human cases [4].

2. Materials and methods

Three kinds of SARS CoV strains: HKU39849(1), TW-1 and FFM-1(2) and their cDNAs were used. S, M, N or E cDNA was transferred into pcDNA 3.1(+) vector and pcDNA 3.1(+)/vs-His Topo (QIAGEN K K, Tokyo, Japan). These genes were expressed in eukaryotic cells and *Escherichia coli*. pcDAN 3.1(+) vector, 50 μg each, containing SARS S, M, N, or E DNA was injected i.m. (M.tibia anterior) into C57BL/6 mice (female, 8 weeks CLEA Japan Inc, Japan) and BALB/c mice (female, 8 weeks) three times, at an interval of 7 days. Neutralizing antibodies against SARS CoV in the serum from the mice immunized with SARS S, M, N or -E DNA vaccines were assayed by use of Vero-E6 cell. CTL activity against SARS CoV was studied using human type II alveolar epithelial cells, T7, expressing SARS antigens [6]. PBL from healthy human volunteers were administered i.p. into IL-2 receptor γ-chain disrupted NOD SCID mice [IL-2R(−/−) NOD-SCID], and SCID-PBL/hu mice were constructed [4]. SARS DNA vaccines at 50 μg were injected i.m. into the SCID-PBL/hu mice. CTL activity of human CD8-positive lymphocytes in the spleen from SCID-PBL/hu was assessed using IFN-γ production and 51Cr-release assay [4,5].

3. Results

3.1. Induction of CTL against SARS CoV by SARS (N) DNA and SARS (M) DNA vaccine

Spleen cells from C57BL/6 mice immunized with SARS-S, -M, -N or -E DNA vaccine were cultured with syngeneic T7 lung cells transfected with S, M, N or E cDNA. pcDNA 3.1(+) SARS (N) DNA vaccine induced significantly CTL activity (IFN-γ production) against N cDNA transfected T7 cells (Fig. 1A). Similarly, SARS M DNA vaccine induced SARS antigen M-specific CTL against T7 cells transfected with SARS M DNA (data not shown).

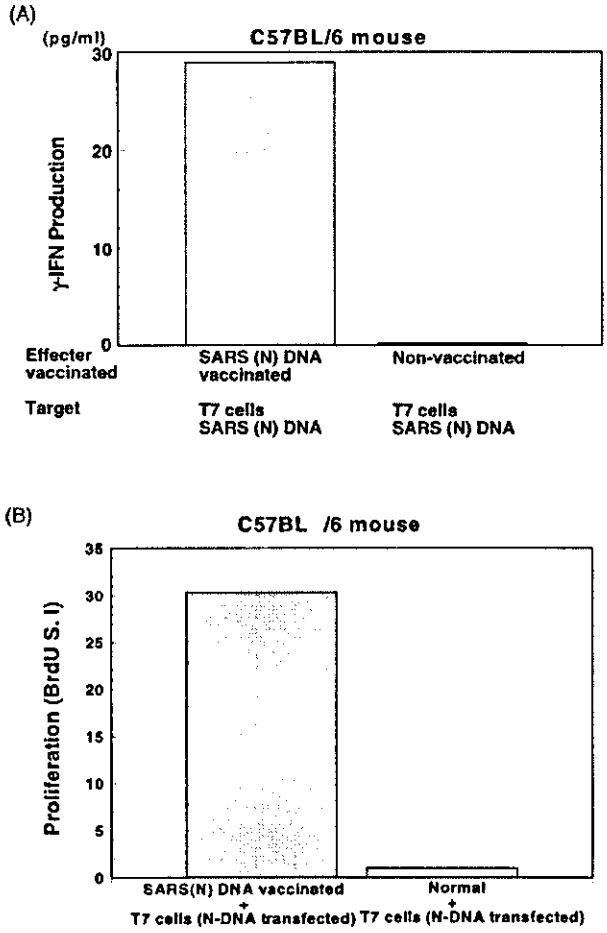

Fig. 1. Induction of CTL and T cell proliferation against SARS (N). (A) Induction of CTL against SARS (N) antigen in the spleen cells from C57BL/6 mice immunized with SARS (N) DNA vaccine. SARS (N) DNA using pcDNA3.1(+) vector was injected i.m. into C57BL/6 mice three times, at an interval of 7 days. CTL activity was assessed by IFN-γ production in the culture of 1×10^6 spleen cells and 1×10^4 T7 lung alveolar type II epithelial cells transfected with SARS (N) DNA at the E/T ratio of 100:1. IFN-γ production was assessed by ELISA assay. (B) Augmentation of lymphocyte proliferation specific for SARS (N) DNA vaccine. 1×10^5 responder cells from vaccinated mice were cultured with Mitomycin C treated 1×10^4 T7 cells transfected with SARS (N) DNA for 48 h and then Bromodeoxy Uridine (BrdU) was added. Proliferative responses were assessed by BrdU assay.

3.2. Augmentation of lymphocyte proliferation specific for SARS CoV antigens by the immunization with SARS (M) DNA and SARS (N) DNA vaccine

The proliferation of splenic T cells stimulated by co-culture either with T7 cells transfected with M DNA or SARS M peptide (TW1 M102-116) was strongly augmented by M DNA vaccine (data not shown). SARS N DNA vaccine also induced proliferation of splenic T cells in the presence of recombinant N protein as well as N DNA-transfected T7 cells (Fig. 1B). Thus, both SARS N DNA vaccine and M DNA vac-

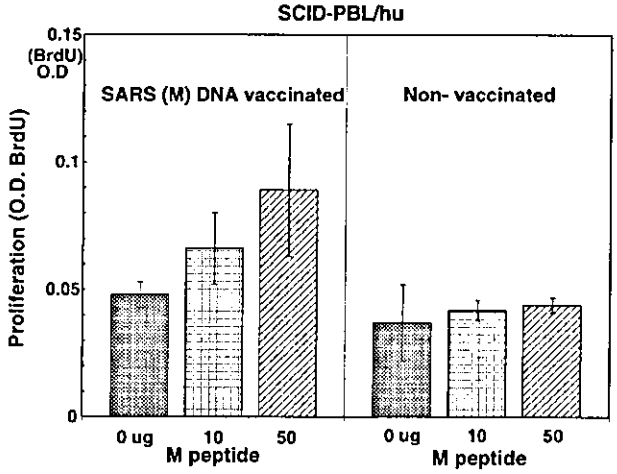

Fig. 2. SARS (M) DNA vaccine induces in vivo human T cell proliferation against SARS CoV in the SCID-PBL/hu human immune systems. 4×10^7 PBL from healthy human voluntcers were administered i.p. into IL-2 receptor γ-chain disrupted NOD SCID mice [IL-2R (−/−) NOD-SCID], and SCID-PBL/hu mice were constructed. Fifty micrograms of SARS DNA vaccine was injected i.m. into these SCID-PBL/hu mice. 1×10^5 spleen cells from these vaccinated mice were cultured with 10~50 μg of SARS M peptide for 3 days. Proliferation was assayed by BrdU.

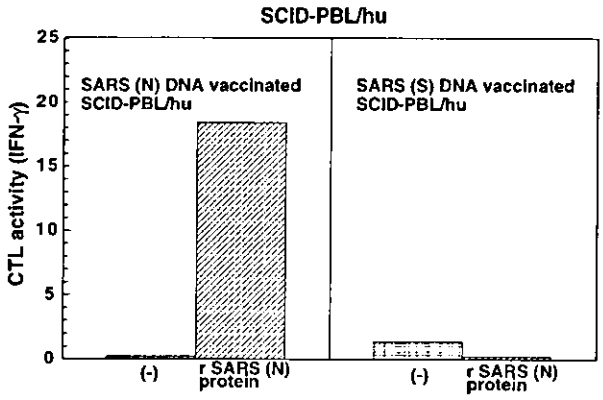

Fig. 3. SARS (N) DNA vaccine induces in vivo human CTL against SARS CoV in the SCID-PBL/hu human immune systems. 4×10^7 PBL from healthy human volunteers were administered i.p. into IL-2 receptor γ-chain disrupted NOD SCID mice [IL-2R (−/−) NOD-SCID], and SCID-PBL/hu mice were constructed 50 μg of SARS (N) DNA vaccine or 50 μg of SARS (S) DNA vaccine. 1×10^5 spleen cells from SCID-PBL/hu were cultured with 10 μg of recombinant SARS (N) protein for 72 h. IFN-γ production in the culture supernatant was assayed using ELISA.

cine were shown to induce T cell immune responses against the relevant SARS CoV antigens.

3.3. SARS M DNA and N DNA vaccines induced human T cell immune responses (CTL and proliferation) in SCID-PBL/hu model

The M DNA vaccine enhanced the CTL activity and proliferation in the presence of M peptide in SCID-PBL/hu mice (Fig. 2). Furthermore, the SARS N DNA vaccine induced CTL activity (IFN-γ production by recombinant N protein or N protein pulsed-autologous B blast cells) and proliferation of spleen cells in SCID-PBL/hu mice (Fig. 3). From these results, it was demonstrated that SARS M DNA vaccine and N DNA vaccine induced human CTL and human T cell proliferative responses.

4. Discussion

We have demonstrated that SARS (M) DNA and (N) DNA vaccines induce virus-specific immune responses (CTL and T cell proliferation) in the mouse systems using type II lung alveolar T cell lines in clone target models [6]. These DNA vaccines induced SARS-CoV-specific CTL and T cell proliferation in vivo human immune systems using SCID-PBL/hu. Gao et al. developed adenovirus based a SARS DNA vaccine encoding S1 polypeptide was capable of inducing neutralizing antibody, while another SARS DNA vaccine encoding N protein generated IFN-γ producing T cells in rhesus monkeys [7]. SARS S DNA vaccine which elicits effective neutralizing antibody responses that generate protective immunity in a mouse model [8]. However its immunogenicity in humans has yet to be established. Therefore, it is very important to evaluate the efficacy of SARS DNA vaccine in a SCID-PBL/hu mice, which is a highly relevant translational model for demonstrating human immune responsiveness. Recently, SARS DNA vaccines capable of inducing human neutralizing antibodies against SARS CoV have been established by our SCID-PBL/hu model. It has been demonstrated that Angiotensin-converting enzyme 2 (ACE2) is a functional receptor for the SARS CoV [9]. A transgenic mouse with human ACE-2 may be useful as an animal model of SARS. Furthermore, ACE-2 transgenic SCID mice should be useful as a human model for pre-clinical trial for SARS vaccines, since ACE-transgenic SCID-PBL/hu model could analyze the human immune responses against SARS infection in vivo. The effect of combination immunization with such SARS vaccines and neutralizing antibody dependent DNA vaccine is now being studied. These DNA vaccines should provide a useful tool for development of protective vaccines.

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